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Stimulated Normal Human Lymphocytes Contain a Ribonuclease-Sensitive DNA Polymerase Distinct from Viral RNA-Directed DNA Polymerase

(70S RNA/RNA tumor viruses/templates/primers)

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ABSTRACT **Ribonuclease-sensitive DNA synthesis is demonstrated in a cytoplasmic particulate fraction of normal human blood lymphocytes stimulated with phytohemagglutinin, but not in unstimulated lymphocytes. DNA polymerase purified from this fraction does not transcribe the heteropolymeric regions of 70S RNA from RNA tumor viruses, thus distinguishing this enzyme from the RNA-directed DNA polymerase (reverse transcriptase) found in oncogenic RNA viruses and human leukemic cells.**

After the discovery of RNA-directed DNA polymerase (reverse transcriptase) in RNA tumor viruses (1–3), we undertook a search in both normal and neoplastic cells for this or a similar enzyme (4–6). Temin and others have suggested that RNA-instructed DNA synthesis may play a role in normal cellular differentiation, gene amplification, and in the secondary immune response (3, 5–9). The possible location of RNA-directed DNA polymerase in the cytoplasm of cells as either a viral or subviral particle, associated with an RNA template/primer, prompted a search for RNase-sensitive DNA synthesis directed by endogenous template/primers in high-speed, partially-purified "pellet" fractions from cytoplasms of mammalian cells (10, 11). Coffin and Temin (10) described such an endogenous RNase-sensitive DNA polymerase reaction in the high-speed cytoplasmic "pellet" of both virus-infected and uninfected rat tissue-culture cells. Ackermann *et al.* (11) described a similar reaction in a continuous cell line established from leukocytes of a patient with leukemia, but this cell line was thought to contain type-C viruses. Recently, RNase-sensitive DNA synthesis has been found in cytoplasmic fractions of *fresh* cells, including normal chick-embryo cells (12) and human leukemic blood leukocytes (13, 14).

Recently, reports have appeared showing that RNA may be a primer for DNA-directed DNA synthesis in bacterial (15, 16) and mammalian (17, 18) systems. Therefore, sensitivity of DNA synthesis to RNase in a complex system such as these cytoplasmic "pellets" (10–14) could be due to RNA-*primed* in contrast to RNA-*directed* DNA synthesis. One way to distinguish between a template and a primer role for RNA is to purify the DNA polymerase(s) from these "pellets" and to analyze the DNA products of any RNA-directed DNA synthesis catalyzed by the purified enzyme(s).

In this communication, we describe the partial purification and properties of a DNA polymerase isolated from a cytoplasmic particulate fraction from normal human-blood lymphocytes stimulated with phytohemagglutinin (PHA). We demonstrate endogenous RNase-sensitive DNA synthesis in the "pellet" fraction, yet the DNA polymerase purified from this fraction has the properties of a DNA-directed DNA polymerase; i.e., this enzyme (*a*) prefers DNA to RNA–DNA hybrid synthetic template/primers and (*b*) does not transcribe heteropolymeric regions of naturally occurring, exogenously supplied single-stranded RNA.

MATERIALS AND METHODS

Preparation of the "Pellet" Fraction. Lymphocytes from normal human peripheral blood were isolated and incubated with or without PHA for 72 hr (5, 19). After incubation, cells were harvested and stored at −70° until use. About 5 g of lymphocytes were thawed and suspended in 25 ml of 10 mM KPO_4 buffer (pH 7.7)–2 mM $MgCl_2$. All subsequent procedures were performed at 0–4°. Suspended cells were centrifuged at 1000 × *g* for 10 min. The resulting pellet was suspended in 25 ml of the same buffer and homogenized with 25–30 strokes in a Dounce homogenizer. The homogenate was diluted with an equal volume of 0.12 M Tris·HCl (pH 8.0)–0.12 M NaCl–0.1 M sucrose. Nuclei and intact cells were removed by centrifugation at 1000 × *g* for 10 min. The resulting cytoplasmic fraction was centrifuged at 60,000 × *g* for 1 hr in a type 40 rotor. The pellet was suspended in 1–2 volumes of 0.1 M Tris·HCl–(pH 8.3)–1 mM dithiothreitol–10% glycerol, sonicated for 1 min at low power in a Branson sonicator, and adjusted to 0.125% (v/v) Triton X-100. This fraction was layered onto a preformed 20–80% glycerol gradient in buffer [10 mM Tris·HCl (pH 7.2)–1 mM dithiothreitol–1 mM EDTA] and centrifuged for 15 hr at 61,000 × *g*. Fractions were collected from the bottom of the tube and assayed for DNA polymerase activity.

DNA Polymerase Assays. The endogenous, RNase-sensitive, DNA synthetic reaction was assayed under the following conditions. An aliquot (90 μl) of fraction 17 of the glycerol gradient (Fig. 1) was incubated at room temperature (24°) for 30 min with either 20 μg of boiled RNase A/ml or with RNase buffer alone (0.01 M Tris·HCl, pH 7.8). The incubation mixtures (150 μl) contained 0.2 M NaCl. After incuba-

Abbreviation: PHA, phytohemagglutinin.

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tion, the volume was increased to 300 μl containing: 50 mM Tris·HCl (pH 7.8); 5 mM $MgCl_2$; 10 mM dithiothreitol; 100 mM NaCl; 0.8 mM each of dATP, dGTP, and dCTP; and 7 μM [^{3}H]TTP (7400 cpm/pmol). At indicated times, 50-μl aliquots were removed and the acid-insoluble radioactivity was determined.

Purification of the Pellet Enzyme. Fractions containing the peak of the endogenous, RNase-sensitive, DNA polymerase activity from the glycerol gradient (Fig. 1*A*) were pooled, brought to a final concentration of 0.5 M NaCl–0.5% Triton X-100, and stirred overnight at 0°. After centrifugation at 100,000 × *g* for 1 hr, the supernatant was diluted with an equal volume of 50 mM Tris·HCl (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol (buffer A). This solubilized extract was loaded onto a column of DEAE-cellulose, and batches were washed with buffer A–0.3 M NaCl. The peak of protein obtained was dialyzed for 5 hr against buffer A. The dialysate (DEAE batch, see Table 1) was absorbed on a phosphocellulose column equilibrated with buffer A. The column was washed with one column volume of 0.1 M NaCl in buffer A, and a linear gradient of NaCl extending from 0.1 to 0.7 M was attached. Fractions were collected and assayed for DNA polymerase activity with activated salmon-sperm DNA and poly(dA-dT) as template/primers. Fractions containing the major peak of activity (eluting at a concentration of 0.35 M NaCl) were pooled and dialyzed against buffer A. The phosphocellulose eluate was then concentrated on a small phosphocellulose column, and layered on a column of Sephadex G-200. The major peak of activity eluted near the void volume; active fractions were pooled and stored in 50% glycerol at −20°.

Enzyme purification and reactions with the partially purified DNA polymerase(s) were assayed in 50-μl reaction mixtures containing the following ingredients: 50 mM Tris·HCl (pH 7.8); 10 mM $MgCl_2$; 80 mM KCl; 5 mM dithiothreitol; 0.08 mM each of dATP, dGTP, and dCTP; 5 μM [^{3}H]TTP (7400 cpm/pmol); and 10 μl of enzyme solution. Added template/primers were at a concentration of 40 μg/ml. Reaction mixtures were incubated at 37° for 30 min. Specific activities do not represent maximum rates, as DNA synthesis was generally not a linear function of time beyond 20 min of incubation.

Cs_2SO_4 Equilibrium Density-Gradient Centrifugation of Endogenous Reaction Product. Standard reaction mixtures as described above (without RNase) were scaled up to 1 ml and incubated at 37° for 3, 10, and 30 min. The reaction products were extracted, purified, and analyzed on Cs_2SO_4 density gradients (14).

RESULTS

Ribonuclease-sensitive endogenous DNA polymerase

Fig. 1*A* shows the results of isopycnic centrifugation in a glycerol gradient of the cytoplasmic particulate fraction from PHA-stimulated lymphocytes. A fraction that bands at a density of 1.095 g/ml contains an endogenous DNA polymerase activity, and this activity is reduced by 80% after prior incubation with RNase (20 μg/ml). Under the conditions used (0.2 M NaCl, pH 7.8), RNase should degrade only single-stranded RNA or short oligoribonucleotides hydrogen-bonded to DNA. In normal blood lymphocytes, *not stimulated by PHA*, we found *no* DNA polymerase activity in the cytoplasmic particulate fraction.

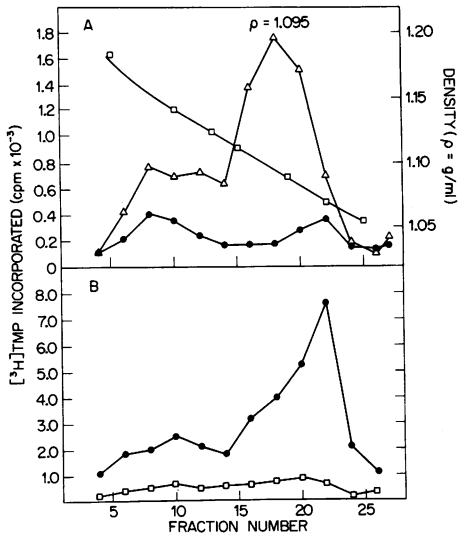

FIG. 1. Isopycnic centrifugation of the cytoplasmic particulate fraction derived from normal lymphocytes (stimulated for 72 hr with PHA, refs. 5 and 19). (*A*) Endogenous DNA polymerase activity. Reaction mixtures were as described in *Methods;* incubation time was 30 min. △——△, endogenous DNA synthesis; ●——●, endogenous DNA synthesis determined after prior incubation of gradient fraction with RNase; □——□, density of fraction measured optically. (*B*) DNA polymerase activity in the presence of $(dA)_n \cdot dT_{10}$ (●——●) and $(A)_n \cdot dT_{12-18}$ (□——□), each at a concentration of 50 μg/ml.

Crude preparations of RNA-directed DNA polymerase from RNA tumor viruses prefer hybrid synthetic template/primers [such as $(A)_n \cdot dT_{12-18}$] to duplex DNA synthetic templates [such as $(dA)_n \cdot dT_{12-18}$] (20). To determine whether the DNA polymerase(s) in the particulate fraction from stimulated lymphocytes share(s) these properties, we assayed for DNA polymerase activity in the presence of these synthetic template/primers. As shown in Fig. 1*B*, $(dA)_n \cdot dT_{10}$ stimulated DNA synthetic activity in every fraction of the isopycnic gradient to a much greater extent than $(A)_n \cdot dT_{12-18}$. This template/primer response is characteristic of cellular polymerases, as opposed to RNA-directed DNA polymerase from RNA tumor viruses (4, 20–23). When activated salmon-sperm DNA was used as template/primer, activity was found in the regions of the endogenous reaction and near the top of the gradient (data not shown). Activity at the top of the gradient represents soluble DNA polymerase, liberated from cellular particulate matter.

Characteristics of the endogenous reaction

Sensitivity of the endogenous reaction to *N*-demethylrifampicin and actinomycin D is shown in Fig. 2*A* and *B*. High concentrations of actinomycin D inhibit DNA-directed DNA synthesis, while *N*-demethylrifampicin (at high concentrations) inhibits the endogenous DNA polymerase reaction of oncornaviruses (25). With high concentrations of actinomycin D, the endogenous DNA polymerase reaction of the lymphocyte "pellet" was inhibited by more than 85% (Fig. 2*B*). This inhibition was significant at early (2 min), as well as late, time points, suggesting that the endogenous DNA synthesis is at least in part directed by a DNA template, or if directed by RNA, a newly synthesized DNA primer may be required. In contrast, actinomycin D inhibits only the late

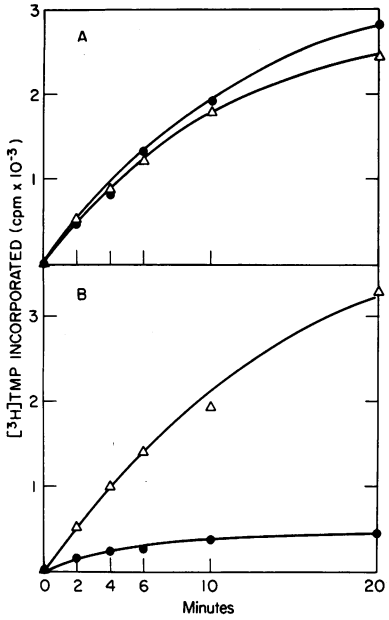

FIG. 2. Effects of *N*-demethylrifampicin and actinomycin D on the endogenous DNA polymerase reaction of the "pellet" fraction from normal lymphocytes (Fig. 1). Reaction conditions were identical to those described in *Methods* (without prior incubation with RNase), except for the following additions: (*A*) To each reaction was added either *N*-demethylrifampicin (300 μg/ml) dissolved in $(CH_3)_2SO$ (●——●) or $(CH_3)_2SO$ alone (△——△). (*B*) To each reaction was added either actinomycin D (50 μg/ml) dissolved in 0.01 M Tris·HCl, pH 7.8 (●——●), or buffer alone (△——△).

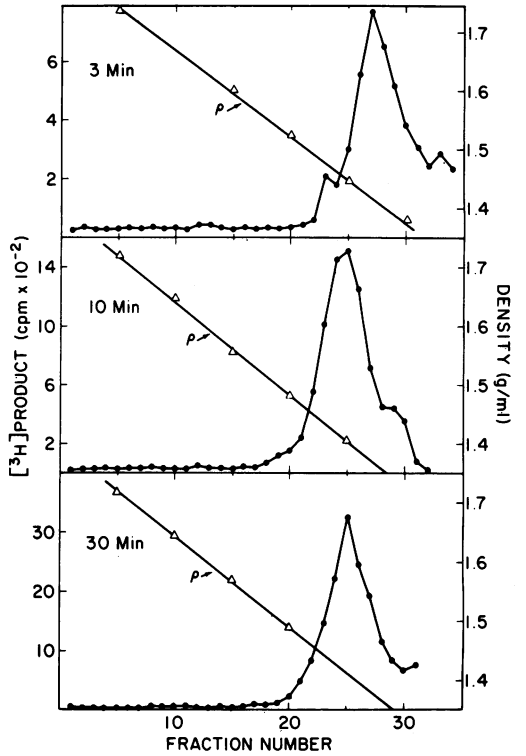

FIG. 3. Equilibrium density-gradient centrifugation of the product of the endogenous, RNase-sensitive DNA polymerase reaction from the 60,000 × *g* cytoplasmic pellet. Cl_3CCOOH-insoluble radioactivity (●——●); fraction density (△——△).

phase of the endogenous polymerase reaction of RNA tumor viruses (26). At concentrations of *N*-demethylrifampicin sufficient to inhibit crude preparations of RNA-directed DNA polymerase (25, 27) or RNA-dependent reactions catalyzed by a DNA polymerase from leukemic cells (5), there was no inhibition of the endogenous, RNase-sensitive DNA synthesis of the "pellet" fraction of normal lymphocytes (Fig. 2*A*).

Nature of the product from endogenous reaction

Analysis of the endogenous-reaction products in density gradients of Cs_2SO_4 is shown in Fig. 3. The density distribution of the product of 3-, 10-, and 30-min incubations from an endogenous reaction (previously shown to be sensitive to RNase) shows that all the radioactivity bands in the DNA region; no material bands at the density of RNA–DNA hybrids at any time point. The absence of RNA–DNA hybrid structures supports the notion that these reactions are not RNA-directed.

It is possible that RNA–DNA hybrids may be formed and subsequently destroyed by RNase activity in the "pellet" fraction from these lymphocytes. Indeed, in one of eight experiments, a small peak of radioactivity was observed at the density of RNA in Cs_2SO_4 density gradients. Consequently, we tested the stability of such hybrids when mixed with the lymphocyte "pellet" fraction: (*a*) Avian myeloblastosis virus was incubated in a standard DNA polymerase reaction mixture alone or mixed with the lymphocyte "pellet" fraction. Equal amounts of RNA–DNA hybrid were recovered on Cs_2SO_4 gradients of the nucleic acids purified from both reaction mixtures. (*b*) Equal portions of purified RNA–DNA hybrid, made from RNA-directed DNA polymerase and 70S RNA both purified from avian myeloblastosis virus, were incubated (30 min at 37°) alone or with the lymphocyte "pellet" fraction. Nucleic acids were then purified and analyzed on Cs_2SO_4 gradients. Partial degradation of RNA–DNA hybrid was observed after incubation with the lymphocyte "pellet" fraction; 50% of the nucleic acids recovered still banded at hybrid density or greater ($\rho > 1.55$). Thus, RNA–DNA hybrids may possibly be formed, and then degraded, during short periods of endogenous DNA synthesis in the lymphocyte "pellet" fraction.

Purification of the pellet enzyme

Glycerol-gradient fractions possessing endogenous, RNase-sensitive DNA-polymerase activity were taken as the source for enzyme, rather than the crude cytoplasmic "pellet," because the isopycnic centrifugation separated soluble, and possibly irrelevant, DNA polymerases from the fraction of interest (Table 1). The "pellet" DNA polymerase was purified as described in *Methods*.

Properties and template characteristics of the partially purified pellet enzyme

Characteristics of the Enzyme. The "pellet" DNA polymerase has an absolute requirement for a divalent cation; manganese can substitute for magnesium. All four deoxynucleoside triphosphates are required for maximal activity with natural template/primer. With $(dA)_n \cdot dT_{10}$ as template, the potassium optimum of the purified enzyme is 80 mM, and the magnesium optimum is 12 mM. A broad optimum of pH, between 7.2 and 8.1, is observed with the same template/primer.

Response to DNA and DNA–RNA Hybrid Templates. Table 2 lists enzyme activity in the presence of magnesium with several natural and synthetic DNA and RNA–DNA hybrid template/primers. $(dA)_n \cdot dT_{10}$ gives the maximal activity. The "pellet" DNA polymerase has relativity low activity with activated DNA. The ratio of activity with $(dA)_n \cdot dT_{10}$ (the preferred template/primer) to $(A)_n \cdot dT_{12-18}$ has ranged between 5 to 1 and 20 to 1.

In contrast, a DNA polymerase purified from a "pellet" fraction of human leukemic lymphoblasts (13, 14) has greater activity with $(A)_n \cdot dT_{12-18}$ than with $(dA)_n \cdot dT_{10}$ in the presence of magnesium. Further, the normal lymphocyte "pellet" DNA polymerase does not transcribe $(C)_n \cdot dG_{12}$, which is an effective template/primer for both viral RNA-directed DNA polymerase (20) and for the leukemic "pellet" DNA polymerase (13, 14).

Response to 70S RNA. In the absence of oligodeoxythymidylate (dT_{12-18}), 70S RNA from avian myeloblastosis virus gives a barely detectable reaction when the radioactive precursor of the highest available specific activity is used; this reaction is insensitive to RNase. If 70S RNA from avian myeloblastosis virus is used as template in the presence of dT_{12-18}, DNA synthesis is stimulated at least 20-fold when [^{3}H]TTP is used as precursor (Table 2). However, the product of this primer-stimulated reaction is not heteropolymeric, as shown by the lack of incorporation of dCTP (Table 2). In contrast, the DNA polymerase from a cytoplasmic particulate fraction from human leukemic lymphoblasts transcribes the *heteropolymeric* regions of 70S RNA from avian myeloblastosis virus, both in the presence and absence of dT_{12-18} (13, 14). Failure of the "pellet" DNA polymerase from normal lymphocytes to transcribe heteropolymeric regions of 70S RNA clearly distinguishes this enzyme from both the leukemic "pellet" DNA polymerase and from RNA-directed DNA polymerase of RNA tumor viruses.

Template Characteristics in the Presence of Manganese. The relative rates of DNA synthesis directed by $(dA)_n \cdot dT_{10}$ and $(A)_n \cdot dT_{12-18}$ have been shown to distinguish normal cellular DNA polymerases from viral RNA-directed DNA polymerase (4, 20–23). Activities with these template/primers were generally measured in the presence of magnesium (20–23).

TABLE 1. *Purification of the DNA polymerase from a "pellet" fraction of normal lymphocytes*

Fraction	Total protein (mg)	Total activity (cpm)	Specific activity (pmol/mg protein)
1. Glycerol gradient peak	42.9	—*	—*
2. Solubilized extract	20.7	1.7×10^8	1077
3. DEAE batch	13.1	1.3×10^8	1391
4. Phosphocellulose eluate (0.35 M peak)	1.3	6.7×10^7	6985
5. "Sephadex G-200"	0.08	4.5×10^6	9977

The degree of enzyme purification shown above is a minimum estimate, since the purification proceeds from a very small fraction of total cell proteins. An asterisk (*) denotes that activity was not proportional to input of protein.

When manganese is used as the divalent cation with the "pellet" DNA polymerase from normal lymphocytes, the ratio of reaction rates obtained with various template/primers changes markedly (data not shown). Therefore, when comparing viral and cellular DNA polymerases, template/primer characteristics observed in the presence of manganese must be interpreted with caution.

Comparative Properties of DNA Polymerases from Normal Lymphocytes. We describe elsewhere two DNA polymerases, termed DNA polymerase I and II, purified from normal human lymphocytes (24). The relative activities of these two enzymes and the "pellet" DNA polymerase with several template/primers differ. Maximal DNA synthesis with DNA polymerase I is obtained with activated salmon-sperm DNA and Mg^{++}, while maximal synthesis catalyzed by polymerase II is obtained with $(dA)_n \cdot dT_{10}$ and Mn^{++} (24). In contrast, maximal synthesis with the "pellet" DNA polymerase is seen with $(dA)_n \cdot dT_{10}$ and Mg^{++}. *N*-ethylmaleimide (0.1 mM) inhibits activity (assayed with activated DNA) of lymphocyte DNA polymerase I and II by 99 and 12%, respectively. The lymphocyte "pellet" DNA polymerase assayed at about the same protein concentration and with the same template/primer, is intermediate in sensitivity to this compound (55% inhibition). The "pellet" DNA polymerase elutes differently on phosphocellulose from the two other DNA polymerases.

TABLE 2. *Template/primer characteristics of the "pellet" DNA polymerase from normal lymphocytes and activity with template/primers useful in distinguishing RNA-directed DNA polymerase from cellular DNA polymerases*

Template/primer	pmol [^{3}H]TMP incorporated per 10 μg of enzyme
Activated DNA*	7.3
Native DNA	1.7
Denatured DNA	1.7
Poly(dA-dT)	7.9
$(A)_n \cdot (dT)_n$	50
$(dA)_n \cdot dT_{10}$	155
$(A)_n \cdot dT_{12-18}$	32
$(C)_n \cdot dG_{12}$	0
70S RNA (Avian myeloblastosis virus)	<0.1
70S RNA plus RNase A (10 μg/ml)	<0.1
70S RNA plus dT_{12-18}	
[^{3}H]TTP plus dATP, dCTP, and dGTP	10.1
[^{3}H]dCTP plus dGTP, TTP, and dATP	<0.1
[^{3}H]dCTP plus dGTP, TTP, dATP, and RNase A (10 μg/ml)	<0.1

Reaction conditions were as described in *Methods*, except that various templates/primers were used as indicated. With poly(dA-dT) and $(A)_n \cdot (dT)_n$, the substrates were 80 μM dATP and 7.0 μM[^{3}H]TTP (7400 cpm/pmol). With $(C)_n \cdot dG_{12}$ as template/primer, the substrates were 80 μM dCTP and 8.0 μM [^{3}H]dGTP (3730 cpm/pmol). With 70S RNA as template/primer, 4.8 μM [^{3}H]dTTP (23,000 cpm/pmol) or 4.4 μM dCTP (12,500 cpm/pmol) was used. 70S RNA and dT_{12-18} were used at a concentration of 1.25 μg per reaction mixture (50 μl).

* Optimal activation of this template/primer was tested by assay with lymphocyte DNA polymerase I (24).

Although this evidence suggests that the "pellet" DNA polymerase may be distinct from the other two cellular DNA polymerases, further information is required to establish this point conclusively.

DISCUSSION

The DNA polymerase reaction present in the cytoplasmic "pellet" has several characteristics: (*a*) The endogenous reaction is sensitive to RNase; (*b*) equilibrium density-gradient centrifugation of the product of the endogenous reaction fails to consistently demonstrate RNA–DNA hybrids; (*c*) when purified, the DNA polymerase from this fraction has all of the properties of a DNA-directed DNA polymerase. For example, this enzyme prefers $(dA)_n \cdot dT_{10}$ to $(A)_n \cdot dT_{12-18}$ as template/primer, does not transcribe the heteropolymeric regions of viral 70S RNA, and will not accept $(C)_n \cdot dG_{12}$ as template/primer.

There are two interpretations of these data: (*a*) The endogenous DNA synthesis in the "pellet" may be directed by RNA, and the failure to find RNA–DNA hybrids among the products is due to the presence of RNase in the "pellet" that destroys the RNA in such a hybrid (28). The purified "pellet" DNA polymerase may require a specific endogenous RNA present in the pellet. However, template/primer specificity of this nature is not characteristic of either RNA-directed DNA polymerase of RNA tumor viruses (29) or of human leukemic cells (13, 14). If this interpretation were correct, our data could be taken as support for Temin's protovirus theory (3). (*b*) Another interpretation is that the RNA in the "pellet" acts as a primer molecule for DNA-directed DNA synthesis. [About 6% of the nucleic acids in the "pellet" bands at the density of DNA ($\rho < 1.50$) in Cs_2SO_4 density gradients.] RNase, by destroying an RNA primer, may inhibit the DNA-directed reaction. We cannot exclude either of these interpretations with the available data.

There is a remarkable similarity in the characteristics of DNA-synthesizing cytoplasmic particulate fractions in our system (PHA-stimulated blood lymphocytes from normal human adults) and in normal, "virus-free" chicken embryos, described by Kang and Temin (12). These systems are not the same as the RNA-directed DNA polymerase of RNA tumor viruses, a point emphasized by Kang and Temin as well (12), or the RNA-directed DNA polymerase of human leukemic cells (13, 14). Further experiments are required to clarify the mechanism of RNase-sensitive DNA synthesis in these normal cytoplasmic systems.

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